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14. ABSTRACT

In response to a variety of different stressors, cells initiate a death program that is carried out by a family of cysteine proteases known as caspases. Caspase activation in response to chemotherapeutics typically proceeds through pathways that induce transit of cytochrome c from the intermembrane space of the mitochondria to the cytoplasm. Engagement of a cytosolic protein, Apaf-1, by cytochrome c nucleates the formation of a structure known as the apoptosome, in which caspase 9 is activated by Apaf1/cytochrome c. For many tumor types (eg. ovarian, leukemias, prostate cancers), chemotherapy fails either because the agents employed fail to trigger the release of mitochondrial cytochrome c or because the apoptosome is in some way defective and so cannot respond to cytochrome c. However, in analyzing a large battery of breast cancer cell lines, we made the surprising discovery that they were exquisitely sensitive to cytochrome c, dying much more rapidly than normal breast cells in response to even low levels of cytoplasmic cytochrome c. Why then are breast cancers not rapidly and uniformly killed by chemotherapeutics? Our initial analysis indicates that breast cancer cells are highly variable in their ability to release cytochrome c following treatment with chemotherapeutic agents, despite their uniform susceptibility to cytochrome c once it has appeared in the cytoplasm. These findings suggest that treatments able to bypass the mitochondria and activate the apoptosome directly (ie. mimic mitochondrially-released cytochrome c) might be more effective than conventional therapeutics in inducing the death of breast cancer cells. This report documents our initial attempts to access this pathway therapeutically.

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Introduction

The premise of our proposal derived from our findings that breast cancer cells were more sensitive to cytochrome c-induced apoptosis than their normal counterparts. Based on these findings, we wished to develop cytochrome c mimetic therapeutics for the treatment of chemoresistant breast tumors. For this purpose, we wished both to modify cytochrome c itself as a potential therapeutic and screen for small molecules that might mimic cytochrome c.

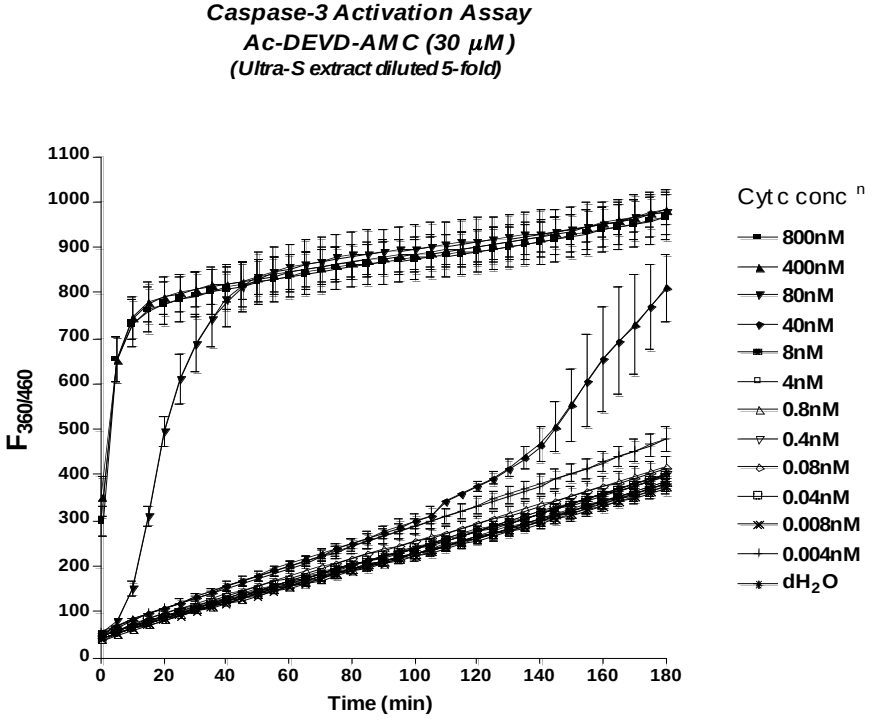
Our original statement of work contained the following aims. For practical reasons, we have reversed the order of the aims and have begun with Task 3 to identify small molecule apoptosome activators (the tasks are not interdependent).

Task 1: Construction of cytochrome c and heme lyase variants allowing the production of cytoplasmically active cytochrome c (months 1-6)

Task 2: Testing of cytoplasmic cytochrome c/heme lyase in tissue culture and mice (months 12-20)

Task 3: Identification and testing of small molecule post-mitochondrial caspase activators (months 21-36)

As detailed in our original proposal, our first task was to prepare large amounts of cytosol and miniaturize the assay to make it feasible to screen large numbers of compounds robotically. We successfully calibrated the assay using cyt c itself as a caspase activator, as shown in Fig 1 and have decided to run a long term assay (3 hours) to enable us to pick up even weak activators that might provide clues for building appropriate more potent derivatives.



The assay shown in Fig. 1 uses a fluorometric indicator of caspase 3 activity, which we are now using. However, in an initial series of screens we used a colorimetric indicator. We have found this to be both less sensitive, and less accurate than the fluorometric substrate (in part because some of the compounds being screened are colored and give a false signal). That said, we picked up several potentially interesting compounds in our first screen based on their apparent ability to activate caspases in the absence of mitochondria (Fig. 2). It is not clear that these are valid "hits" however and need to be verified in the fluorometric assay. We are continuing with our screening and compound characterization in the coming year. We have also recently begun engineering the plasmids required for the work in Tasks 1 and 2. In particular, we are modifying a bacterial expression system that will allow us to produce Heme lyase and Tat-tagged cytochrome c together in bacteria. This should allow us to produce Heme-modified cytochrome c for direct delivery to cells.

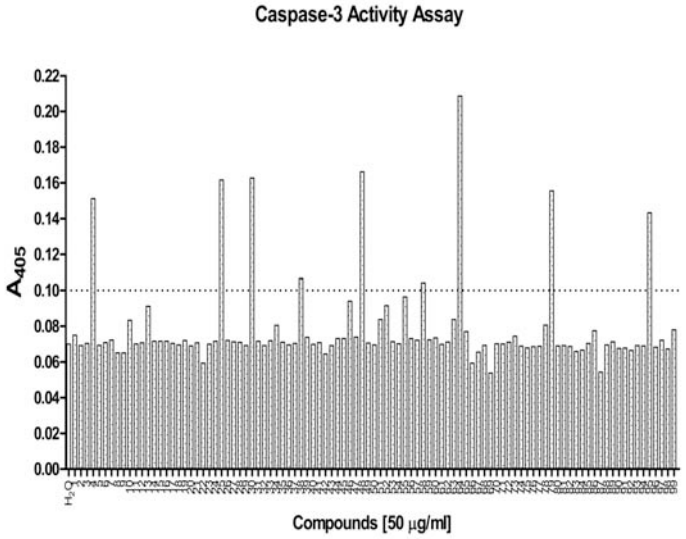


Figure 2. A series of compounds from the small molecule library were added to purified cytosol, incubated, and assayed for caspase 3 activity. A series of possible "hits" promoting activity above baseline are shown.

Key Research accomplishments

Developed and refined screen for cytochrome c mimetics
Screened first 6000 compounds
Identified several initial compounds that will merit further testing

Reportable outcomes
None yet, but work is well underway.

Conclusion We have initiated our screen and have begun construction of the cytochrome c and heme lyase variants described in Task 1.